TRANSGENIC PLANTS MODIFIED TO CONTAIN RESVERATROL GLUCOSIDE AND USES THEREOF

TECHNICAL FIELD OF INVENTION

This invention relates to transgenic plants and plant cells modified to

5   accumulate resveratrol glucoside or to contain increased levels thereof.

BACKGROUND OF THE INVENTION

Stilbenes are biologically active phenolic compounds exhibiting a broad
spectrum of antibiotic and pharmacological activities, and plants modified with
respect to stilbene content may useful for various purposes. Diverse plants and

10 plant families naturally produce stilbenes, including grape (Vitaceae), Scots pine
(Cyperaceae), tall fescue (Poaceae), and peanut (Arachaceae) (Sotheeswaran, S.
and Pasupathy, V. 1993. "Distribution of resveratrol oligomers in plants,"
Phytochemistry 32:1083-1092; Powell, et al. 1994. "Isolation of resveratrol from
Festuca versuta and evidence of the widespread occurrence of this stilbene in the

15 *Poaceae*," *Phytochemistry* 35:335-338; Ingram, J.L. 1976. "3,5,4'-
Trihydroxystilbene as a phytoalexin from groundnut (*Arachis hypogaea*).
Phytochemistry 15,1791-1793).

Stilbenes have been reported to play a role in plant resistance to fungal
pathogens. Constitutive stilbene accumulation is believed to function as a

20 mechanism of general resistance to microbial pathogens, while in some plants,
stilbenes accumulate as phytoalexins in response to microbial attack.

Further, stilbenes are thought to have health-promoting effects, and plants
containing stilbenes may be desirable in human and animal diets. Stilbenes have
been shown to have a number of beneficial effects on human health, based on

25 epidemiological studies and laboratory studies involving humans, animals, cell
cultures and enzyme assays (Jang, et al. 1997. "Cancer chemopreventative activity
of resveratrol, a natural product derived from grapes," *Science* 275:218-220).

Of particular interest is the stilbene resveratrol. Resveratrol is present in wine and may be involved in the health-promotive effects of moderate wine consumption. The increased consumption of resveratrol has been proposed as a way to reduce cancer rates and coronary heart disease in humans (Soleas, et al.

5   1997. "Wine as a biological fluid: history, production, and role in disease prevention," *J Clin Lab Anal* 11: 287-313). Resveratrol and plant extracts containing resveratrol have been shown to be effective in prevention and therapy of atherosclerosis (Arichi, et al. 1982. *Chem Pharm Bull* 30:1766), as an anti-inflammatory agent (Kimura, et al. 1985. *Biochem Biophys Acta* 834:275), and as

10   an anti-hyperoxidative agent (Kimura, et al. 1983. *Plant Med J Med Plant Res* 49:51). Resveratrol showed significant inhibition of aberrant colon crypt formation in a carcinogen (azoxymethane) treated rat model, suggesting utility in inhibiting tumorogenesis in humans (Steele, et al. 1998. "Cancer chemoprevention drug development strategies for resveratrol," *Pharm Bio* 36:62-68 suppl).

15   Resveratrol has also been found to promote the formation of nitroxides which are effective as vasodilatory agents and in inhibiting platelet aggregation (Fitzpatrick, et al. 1993. *Am J Physiol* 34:774).

Resveratrol has the following chemical structure:

$$HO-\langle\rangle-CH=CH-\langle\rangle\begin{matrix}OH\\\\OH\end{matrix}$$
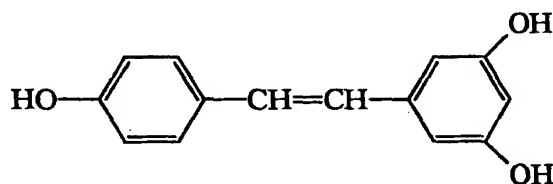
20   The biosynthetic pathway of resveratrol in plants involves stilbene synthase. Resveratrol is formed when stilbene synthase converts one molecule of p-coumaroyl-CoA and three molecules of malonyl-CoA into resveratrol, i.e., 3,5,4'-trihydroxystilbene. In some plant species, resveratrol production is inducible, and resveratrol accumulates as a phytoalexin following microbial attack (Langcake, P.

25   and Pryce, R.J.1976. "The production of resveratrol by *Vitis vinifera* and other members of the Vitaceae as a response to infection or injury," *Physiol Plant*

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Pathol 9:77-86; Dercks, et al. 1995. "Stilbene phytoalexins and disease resistance in *Vitis.*" In *Handbook of Phytoalexin Metabolism and Action*, M. Daniel and R.P. Purkayastha, eds, Marcel Dekker, Inc. USA, pp. 287-315).

5   Not all plants naturally accumulate resveratrol or other stilbenes. Heterologous accumulation of resveratrol has been achieved in plants which do not naturally accumulate resveratrol by genetic engineering to express resveratrol synthase. For example, transformation of tobacco with a resveratrol synthase (*RS*) genomic clone from peanut resulted in the rapid accumulation of resveratrol following treatment of cell suspension cultures with fungal elicitor (Hain, et al.
10 1990. "Expression of a stilbene synthase gene in *Nicotiana tobacum* results in synthesis of the phytoalexin resveratrol," *Plant Mol Biol* 15:325-335). Subsequent experiments with an *RS* gene (*Vst1*) from *V. vinifera* L. demonstrated a significant level of resistance in transgenic tobacco, tomato, and rice to fungal pathogens *Botrytis cinerea, Phytophthora infestans*, and *Pyricularia oryzae*,
15 respectively [Hain et al. 1993. "Disease resistance results from foreign phytoalexin expression in a novel plant," *Nature* 361:153-156; Thomzik, et al. 1997. "Synthesis of a grapevine phytoalexin in transgenic tomatoes (*Lycopersican esculetum* Mill.) conditions resistance against *Phytophthora infestans*," *Physiol Mol Plant Path* 51:265-278; Stark-Lorenzen, et al. 1997. "Transfer of a grapevine
20 stilbene synthase gene to rice (*Oryza sativa* L.)," *Plant Cell Rep* 16:668-673]. These reports demonstrated that accumulation of resveratrol in foreign plant species transformed with a resveratrol synthase gene provides a means by which a broad spectrum of increased resistance to fungal pathogens can be achieved in any plant. Increased resistance to fungal pathogens, in turn, results in increased crop
25 production and reduction in the use of crop protection chemicals.

    Accumulation of resveratrol has also been reported to have some negative side effects in plants. For example, Fischer, et al. reported that plants accumulating increased levels of resveratrol were rendered male sterile. Not only was the pollen destroyed, but the plants also demonstrated lower seed yields and
30 altered flower color (Fischer, et al. 1997. "Stilbene synthase gene expression

causes changes in flower colour and male sterility in tobacco," *Plant J* 11:489-498).

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Resveratrol-3-O-β-D-glucopyranoside (3,4',5-trihydroxystilbene-3-β-D-glucoside, polydatin, piceid; hereinafter referred to as "resveratrol glucoside" or "RGluc") is a resveratrol conjugate. The chemical structure for the trans and cis isomers are given below.
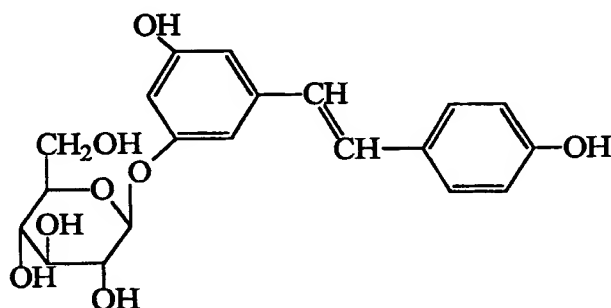
trans isomer
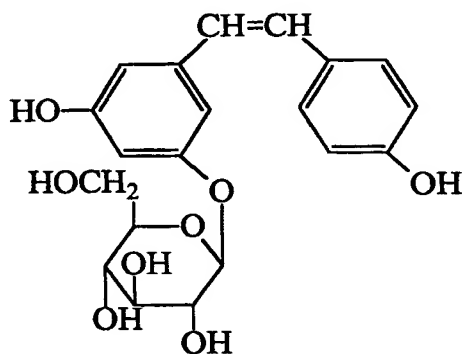
cis isomer

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Both trans- and cis-RGluc have been isolated from nontransgenic plants, for example, grapes (Jeandet, et al. 1994. "Occurrence of a resveratrol-β-D-glucoside in wine: preliminary studies," *Vitis* 33:183-184), *Eucalyptus sideroxylon* wood (Hillis, et al. 1974. "Polyphenols of *Eucalyptus sideroxylon* wood," *Phytochemistry* 13:1591-1595), and Norway spruce (Alcubilla-Martin, M.

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1970. "Extraction, chromatographic separation, and isolation of fungistatic

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substances from the inner bark of Norway spruce," *Z. Pflanzenernaehr. Bodenk.* 127:64-74).

It has been reported that human benefits previously thought to be due to resveratrol are also attributable to RGluc. For example, in International Application WO 9958119, a method for preventing or treating restenosis, a medical condition characterized by recurrent stricture of a heart valve, and for preventing the recurrence or progression of coronary heart disease was provided which involves administration of an active agent comprising cis-resveratrol, trans-resveratrol, a mixture thereof, or a pharmacologically acceptable salt, ester, amide, prodrug, or analog thereof. Both cis- and trans-resveratrol glucoside (RGluc) were listed among the active agents, and the patent discloses these compounds as being either naturally occurring or chemically synthesized in the laboratory.

Subsequent to the epidemiological and biochemical studies which indicated that the resveratrol in wine was at least in part providing valuable protection against cardiovascular disease, numerous labs detected high levels of RGluc in several red and white wines. In view of these findings, it was reported that RGluc likely contributes to the human health benefit of wines (Goldberg, et al. 1996. :Resveratrol glucosides are important components of commercial wines," *Am J Enolog and Viticulture* 47:415-420).

The present invention describes transgenic plants and plant cells that have been modified to contain heterologous RGluc, as well as use of such plants or plant cells in manufacturing foods, nutritional supplements, animal feed supplements, nutraceuticals, and pharmaceuticals to serve as nutritional and therapeutic elements in human and animal diets.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A and 1B (SEQ ID NO:1) depict the sequence of the promoter + resveratrol synthase coding region + terminator cassette (*Hind*III partial digestion fragment) which was transferred into the *Hind*III site of the T-DNA region of

pGA482, preparing it for *Agrobacterium*-based transfer to plant cells. FIG. 1A
contains bases 1-1920, and FIG. 1B contains bases 1921-3089. *Hind*III restriction
enzyme sites are found at bases 1 to 6 and 3084 to 3089 (indicated by "*"). A
*Nco*I restriction enzyme site is found at bases 902 to 907 (indicated by "†"). A

5   *Bam*HI restriction enzyme site is found at bases 2849 to 2854 (indicated by "▪").
Bases 1 to 901 contain the CaMV promoter (with duplicated enhancer element)
and TEV untranslated region from pRTL2. Bases 902 to 2854 contain the *Nco*I
(partial digest) and *Bam*HI (complete digest) restriction enzyme generated
fragment from the RS cDNA clone (indicated by underlining). Bases 904 to 2064

10   contain the coding region of the RS protein (indicated by italics), and the amino
acid sequence is given in SEQ ID NO:2. Bases 2855 to 3089 contain the CaMV
untranslated region from pRTL2.

FIG. 2 is a schematic drawing of the vector constructs used in this method:
double CaMV35S:RS binary vector containing a resveratrol synthase cDNA from

15   peanut.

FIG. 3A and FIG. 3B are a composite photo of the autoradiography results
of Northern blot analysis (RNA get blot analysis) indicating comparative levels of
resveratrol synthase (RS) to chalcone synthase (CHS) transcript levels in the
leaves, internodes and roots. FIG. 3A depicts RS transcript levels total RNA (10

20   µg/lane) extracted from leaves, internodes and roots of three independently
transformed alfalfa lines (lanes 1-3) and one control line (C=pGA482 vector
control plant). FIG. 3B depicts the same total RNA extracts hybridized with an
alfalfa CHS cDNA probe. In both experiments (FIG. 3A and FIG. 3B),
hybridization with an 18S ribosomal RNA probe (18S) served as a loading

25   control.

FIG. 4 is the data from the reverse phase (C18) HPLC analysis of crude
acetone extracts of transgenic alfalfa plants revealing the unknown compound
later identified as RGluc in the CaMV35S:RS transformed alfalfa line
(chromatogram A) that is not present in a pGA482 binary vector control plant

(chromatogram B). RGluc eluted at approximately 8.2 minutes under these conditions.

FIG. 5 is the UV absorbance spectra of the unknown compound later identified as RGluc compared to a known resveratrol standard.

FIG. 6 is the HPLC chromatogram of the putative resveratrol-conjugate following treatment with beta-glucosidase revealing the release of the resveratrol aglycone. Identity was confirmed by co-chromatography with an authentic resveratrol standard.

FIG. 7 depicts a representative alfalfa shoot labeled to illustrate leaf and internode designation used in the analysis of CaMV35S:RS transformed lines (L=leaves; I= internodes).

FIG. 8 is a graph of the results from HPLC quantitation of RGluc in leaves and internodes along shoots from a CaMV35S:RS transformed line revealing the age-and tissue-dependent accumulation of RGluc represented in terms of microgram resveratrol aglycone equivalents per gram of tissue.

FIG. 9 is a composite photo of the autoradiography results of Northern blot analysis (RNA get blot analysis) indicating resveratrol synthase (RS) transcript levels in the leaves and internodes relative to the age or tissue type, compared to an 18S ribosomal RNA probe (18S) served as a locating control (total RNA=10 µg/lane), indicating that the RS cDNA transcript is produced in all of these transgenic alfalfa tissues.

FIG. 10 is a graph depicting the quantitation of necrotic lesion area in leaves of control (pGA482-transformed plant C-F2) and RGluc-accumulating (CaMV 35S-RS construct transformed plant 35S:RS-D1) plants after wound-inoculation with the alfalfa fungal pathogen *Phoma medicaginis*. The points show the average necrotic lesion size (mm^2) surrounding the wound site at four different inoculum levels (CFU/ml) measured at 10 days post-inoculation in the

transformed CaMV35S:RS and pGA482 vector control plants. The error bars represent the standard deviation of the mean.

FIG. 11 is a graph depicting the quantitation of necrotic lesion area in leaves of control (pGA482-transformed plant C-A1.2) and RGluc-accumulating

5   (CaMV35S-RS construct transformed plant 35S:RS-E2.1) plants after wound-inoculation with the alfalfa fungal pathogen *Phoma medicaginis*. The points show the average necrotic lesion size (mm^2) surrounding the wound site at four different inoculum levels (CFU/ml) measured at 10 days post-inoculation in the transformed CaMV35S:RS and pGA482 vector control plants. The error bars

10   represent the standard deviation of the mean.

FIG. 12A - FIG. 12D is a composite photograph of leaves of RGluc-accumulating plants and the control alfalfa after wound-inoculation with the alfalfa fungal pathogen *Phoma medicaginis* showing the development of fungal necrotic lesions and reproductive structures (pycnidia). FIG. 12A depicts a

15   pGA482 vector control plant leaf at 10 days post-inoculation (CFU/ml=10,000). FIG. 12B depicts a transformed CaMV35S:RS RGluc-accumulating plant leaf at 10 days post-inoculation (CFU/ml=10,000). FIG. 12C depicts trypan blue staining of the inoculated control line showing numerous pycnidia (dark roundish spots) and hyphae (thin dark lines radiating outward from inoculation hole).

20   Wound-inoculated leaves were detached from stems after 10 days then placed at 100% relative humidity for three days before staining. FIG 12D depicts trypan blue staining of the inoculated CaMV35S:RS RGluc-accumulating line showing no pycnidia or hyphae beyond the inoculation wound site. Wound-inoculated leaves were detached from stems after 10 days then placed at 100% relative

25   humidity for three days before staining.

FIG. 13A and 13B are graphs comparing the extent of hyphal ingress to the number of pycnidia in control alfalfa leaves (FIG. 13A) and RGluc CaMV5S:RS-transformed leaves (FIG. 13B). Following trypan blue staining, the total area of hyphal growth was measured and the number of pycnidia scored in

inoculated leaves (CFU/ml=10,000) from the control and CaMV35S:RS lines. For this experiment, the number of lesions measured for control and CaMV35S:RS were n=50 and n=57, respectively.

5   FIG. 14 is a HPLC chromatogram of an extract from a transgenic soy callus transformed with the construct described in Fig. 2. The RGluc peak is marked by "*".

FIG. 15 is a HPLC chromatogram of an extract from a non-transgenic soy callus.

10   FIG. 16 is the ultra-violet diode array scan of the soybean callus-generated RGluc peak.

FIG. 17 is a HPLC chromatogram which demonstrates RGluc partially purified from transgenic soybean callus before treatment with beta-glucosidase.

FIG. 18 is a HPLC chromatogram which demonstrates partially purified RGluc after brief treatment with beta-glucosidase.

15   **SUMMARY OF THE INVENTION**

In one aspect, the present invention is a food comprising edible transgenic plant material capable of being ingested for its nutritional value, wherein the transgenic plant is transformed with a portion of a resveratrol synthase gene and exhibits increased levels of resveratrol glucoside when compared to levels of

20   resveratrol glucoside in plants of the same species which do not comprise the portion of a resveratrol synthase gene. The transgenic plant can be transformed with a resveratrol synthase gene construct. Exemplary transformed plants include legumes. Exemplary legumes include alfalfa and soybean.

In another aspect, the present invention is a food comprising resveratrol

25   glucoside isolated from an edible transgenic plant which is transformed with a portion of a resveratrol synthase gene and exhibits increased levels of resveratrol

glucoside when compared to levels of resveratrol glucoside in plants of the same species which do not comprise the portion of a resveratrol synthase gene. The transgenic plant can be transformed with a resveratrol synthase gene construct. Exemplary transformed plants include legumes. Exemplary legumes include

5   alfalfa and soybean.

In another aspect, the present invention is an edible composition comprising at least a portion of an edible transgenic plant which is transformed with a portion of a resveratrol synthase gene and exhibits increased levels of resveratrol glucoside when compared to levels of resveratrol glucoside in plants of

10   the same species which do not comprise the portion of a resveratrol synthase gene, wherein the composition is suitable for ingestion as a food stuff, a nutritional supplement, an animal feed supplement, or a nutraceutical. The transgenic plant can be transformed with a resveratrol synthase gene construct. Exemplary transformed plants include legumes. Exemplary legumes include alfalfa and

15   soybean.

In another aspect, the present invention is a composition comprising resveratrol glucoside suitable for administration as a food stuff, a nutritional supplement, an animal feed supplement, a nutraceutical, or a pharmaceutical, wherein the resveratrol glucoside is isolated from at least a portion of an edible

20   transgenic plant which is transformed with a portion of a resveratrol synthase gene and exhibits increased levels of resveratrol glucoside when compared to levels of resveratrol glucoside in plants of the same species which do not comprise the portion of a resveratrol synthase gene. The transgenic plant can be transformed with a resveratrol synthase gene construct. Exemplary transformed plants include

25   legumes. Exemplary legumes include alfalfa and soybean.

In another aspect, the present invention is an edible transgenic plant transformed with a portion of a resveratrol synthase gene, wherein the transformed plant exhibits increased levels of resveratrol glucoside when compared to levels of resveratrol glucoside in plants of the same

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species which do not comprise the portion of a resveratrol synthase gene. The transgenic plant can be transformed with a resveratrol synthase gene construct.

5   In another aspect, the present invention is seed from an edible transgenic plant transformed with a portion of a resveratrol synthase gene, wherein the transformed plant exhibits increased levels of resveratrol glucoside when compared to levels of resveratrol glucoside in plants of the same species which do not comprise the portion of a resveratrol synthase gene. The transgenic plant can be transformed with a resveratrol synthase gene construct.

10   In another aspect, the present invention is progeny from an edible transgenic plant transformed with a portion of a resveratrol synthase gene, wherein the transformed plant exhibits increased levels of resveratrol glucoside when compared to levels of resveratrol glucoside in plants of the same species which do not comprise the portion of a resveratrol synthase gene. The transgenic 15   plant can be transformed with a resveratrol synthase gene construct.

In another aspect, the present invention is progeny from seed of an edible transgenic plant transformed with a portion of a resveratrol synthase gene, wherein the transformed plant exhibits increased levels of resveratrol glucoside when compared to levels of resveratrol glucoside in plants of the same species 20   which do not comprise the portion of a resveratrol synthase gene. The transgenic plant can be transformed with a resveratrol synthase gene construct.

In another aspect, the present invention is a method of increasing the nutritional value of an edible plant by transforming the plant with a portion of a resveratrol synthase gene, wherein the transformed plant exhibits increased levels 25   of resveratrol glucoside when compared to levels of resveratrol glucoside in plants of the same species which do not comprise the portion of a resveratrol synthase gene. The transgenic plant can be transformed with a resveratrol synthase gene construct.

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In another aspect, the present invention is a method of using an edible transgenic plant transformed with a portion of a resveratrol synthase gene, wherein the plant exhibits increased levels of resveratrol glucoside when compared to levels of resveratrol glucoside in plants of the same species which do

5   not comprise the portion of a resveratrol synthase gene, to provide a nutraceutical benefit to a human or animal administered the resveratrol glucoside. The transgenic plant can be transformed with a resveratrol synthase gene construct. Preferably, the resveratrol glucoside is administered by ingestion of at least a portion of the plant. Alternately, the resveratrol glucoside is administered by

10   ingestion of a composition comprising resveratrol glucoside isolated from the plant.

In another aspect, the present invention is a method of using resveratrol glucoside isolated from an edible transgenic plant transformed with a portion of a resveratrol synthase gene, wherein the plant exhibits increased levels of

15   resveratrol glucoside when compared to levels of resveratrol glucoside in plants of the same species which do not comprise the portion of a resveratrol synthase gene, to provide a pharmaceutical benefit to a patient administered the resveratrol glucoside. The transgenic plant can be transformed with a resveratrol synthase gene construct.

20   In another aspect, the present invention is a method for creating a transgenic plant comprising an increased level of resveratrol glucoside, comprising transforming a plant with a portion of a resveratrol synthase gene to form a transgenic plant, wherein the transgenic plant exhibits increased levels of resveratrol glucoside when compared to levels of resveratrol glucoside in plants of

25   the same species which do not comprise the portion of a resveratrol synthase gene. Preferably, the plant is transformed by introducing an expression cassette comprising a transcribable DNA fragment, wherein the DNA fragment comprises resveratrol synthase cDNA. Exemplary transformed plants include legumes. Exemplary legumes include alfalfa and soybean.

In another aspect, the present invention is a method of increasing disease resistance in an edible plant by transforming the plant with a portion of a resveratrol synthase gene, wherein the transformed plant exhibits increased levels of resveratrol glucoside when compared to levels of resveratrol glucoside in plants

5   of the same species which do not comprise the portion of a resveratrol synthase gene. The transgenic plant can be transformed with a resveratrol synthase gene construct.

In another aspect, the present invention is a method of decreasing spoilage of an edible plant or plant part after harvesting by transforming the plant or plant

10   part with a portion of a resveratrol synthase gene, wherein the transformed plant or plant part exhibits increased levels of resveratrol glucoside when compared to levels of resveratrol glucoside in plants of the same species which do not comprise the portion of a resveratrol synthase gene. The transgenic plant can be transformed with a resveratrol synthase gene construct.

15   In another aspect, the present invention is an edible transgenic plant comprising at least one recombinant DNA sequence encoding a portion of a resveratrol synthase gene, wherein the plant, upon expression of the gene, exhibits increased levels of resveratrol glucoside when compared to levels of resveratrol glucoside in plants of the same species which do not comprise said recombinant

20   DNA sequence.

In another aspect, the present invention is a seed from an edible transgenic plant comprising at least one recombinant DNA sequence encoding a portion of a resveratrol synthase gene, wherein the plant, upon expression of the gene, exhibits increased levels of resveratrol glucoside when compared to levels of resveratrol

25   glucoside in plants of the same species which do not comprise the same recombinant DNA sequence.

In another aspect, the present invention is progeny from an edible transgenic plant comprising at least one recombinant DNA sequence encoding a portion of a resveratrol synthase gene, wherein the plant, upon expression of the

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gene, exhibits increased levels of resveratrol glucoside when compared to levels of resveratrol glucoside in plants of the same species which do not comprise the same recombinant DNA sequence.

5   In another aspect, the present invention is progeny from seed of an edible transgenic plant comprising at least one recombinant DNA sequence encoding a portion of a resveratrol synthase gene, wherein the plant, upon expression of the gene, exhibits increased levels of resveratrol glucoside when compared to levels of resveratrol glucoside in plants of the same species which do not comprise the same recombinant DNA sequence.

10   In another aspect, the present invention is the use of an edible transgenic plant transformed with a portion of a resveratrol synthase gene, wherein the plant exhibits increased levels of resveratrol glucoside when compared to levels of resveratrol glucoside in plants of the same species which do not comprise the portion of a resveratrol synthase gene, for the preparation of a nutraceutical

15   preparation for achieving a nutritional effect. The transgenic plant can be transformed with a resveratrol synthase gene construct.

          In another aspect, the present invention is the use of an edible transgenic plant transformed with a portion of a resveratrol synthase gene, wherein the plant exhibits increased levels of resveratrol glucoside when compared to levels of

20   resveratrol glucoside in plants of the same species which do not comprise the portion of a resveratrol synthase gene, for the preparation of a pharmaceutical preparation for achieving a therapeutic effect. The transgenic plant can be transformed with a resveratrol synthase gene construct.

DETAILED DESCRIPTION

25   The present invention provides for transformation of plants or plant cells which do not normally accumulate stilbenes with a portion of a resveratrol synthase gene wherein the transgenic plants exhibit increased levels of resveratrol glucoside ("RGluc") when compared to levels of resveratrol glucoside in plants of

the same species which do not comprise that same portion of a resveratrol synthase gene. Such plants include dicots and monocots, including but not limited to alfalfa, soybean, tomato, lettuce, tobacco, corn, maize, cotton, squash, beans and other legumes, melons, broccoli and other cole crops, stone fruits, citrus

5   fruits, and strawberries. In the present invention, unless otherwise stated, as used herein, the term "plant" or "progeny" includes plant parts, plant tissue, plant cells, plant protoplasts, plant cell tissue cultures from which plants can be regenerated, plant calli, plant clumps, explants, plant cells that are intact in plants, or parts of plants, such as embryos, pollen, ovules, flowers, capsules, stems, leaves, seeds,

10   roots, root tips, and the like.

A transgenic plant which has accumulated RGluc is useful in improving human and animal nutrition. Edible transgenic plants high in RGluc can be utilized as food for humans and animals. Edible compositions high in RGluc can also be made by incorporation of the transgenic plants or plant materials, or by

15   incorporation of RGluc isolated from the transgenic plants. Compositions useful for administration as a food stuff, a nutritional supplement, an animal feed supplement, a nutraceutical, or a pharmaceutical can be made by incorporation of the transgenic plants or plant materials, or by incorporation of RGluc isolated from the transgenic plants. The nutritional value of a plant can be increased by

20   transforming the plant with a portion of a resveratrol synthase gene and, as a result, accumulating high amounts of RGluc in the plant. Disease resistance can be increased in the transgenic plant by the accumulation of high levels of RGluc upon expression of a resveratrol synthase gene. Likewise, spoilage of transgenic plants or plant parts can be decreased by the accumulation of high levels of RGluc

25   upon expression of a resveratrol synthase gene.

Preferably, RGluc is administered orally by directly ingesting the transgenic plant. Alternatively, RGluc can be isolated from transgenic plants to be used as a crude extract or purified compound. Administration of transgenically produced RGluc to humans or animals provides enhanced pharmaceutical and

30   nutraceutical effects, including but not limited to benefits received from an

antioxidant, platelet aggregation inhibitor, arachidonate metabolism inhibitor, protein kinase inhibitor, inducer of apoptotic cell death in tumor cells, estrogen receptor antagonist, inhibitor of ribonucleotide reductase, tumor initiation inhibitor, tumor promotion inhibitor, tumor progression and differentiation

5   inhibitor, inhibitor of COX-2 and COX-2 induction, lipoprotein synthesis and release modulator as well as other beneficial effects. Transgenically-produced RGluc is also useful as a ultraviolet light protectant, an ultra-violet stabilizer for biocontrol agents, and an agent in the storage of photochemical energy. Based upon RGluc's role in conferring resistance to plants against fungal pathogens,

10   transgenically produced RGluc can also have applications as an antifungal and antibacterial agent in humans.

Transgenically produced RGluc confers disease resistance to heterologous plants that otherwise do not produce stilbenes or do not constitutively accumulate RGluc and do not, therefore, enjoy the benefits of stilbenes. A plant

15   transgenically altered to comprise a resveratrol synthase gene can generate and accumulate RGluc prior to adverse conditions, including but not limited to infection by a pathogen. Thus, RGluc can be used to improve crop yield and quality of food and other plant products.

The structural differences between RGluc and free resveratrol provides

20   increased chemical stability in RGluc. In particular, the glucose on the 3-hydroxyl of RGluc reduces the potential for oxidation of the aglycone by acting as a protecting group for this hydroxyl and reducing the oxidation potential of the dioxygenated ring, relative to the analogous portion of resveratrol. Consequently, RGluc is less subject to oxidation than is resveratrol. RGluc's decreased potential

25   for oxidation provides advantages over resveratrol both in vitro and in vivo: (1) decreased oxidation by air resulting in increased storage stability in vitro; (2) decreased oxidation by peroxidases, oxygen, peroxides, and other processes in living plant cells, potentially resulting in improved disease resistance in transgenic plants; and (3) decreased oxidation by peroxidases, oxygen, peroxides, and other

30   processes during consumption by humans or animals, potentially resulting in

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improved health benefits. In plants, glycosylated compounds are also stored in plant vacuoles, leading to further stability of RGluc against oxidation.

RGluc is also preferable to free resveratrol in human and animal diets. Under certain conditions, it is believed that RGluc can be biologically active, or 5 can be readily hydrolyzed into the biologically active form. RGluc is readily convertible to resveratrol by beta-glucosidase, by other glucosidases, or by non-enzymatic hydrolysis, such as by acid hydrolysis. It is believed that RGluc will be absorbed in the digestive system as readily or more readily than free resveratrol, as is the case for numerous sugar derivatives of pharmaceuticals in comparison to 10 the corresponding aglycones. It is further believed that RGluc is hydrolyzed to free resveratrol before or after absorption in the mammalian digestive tract. Therefore, plants which contain RGluc provide a stable dietary source of resveratrol.

The methods of producing RGluc from transgenic plants or cells from 15 transgenic plants as presented herein provide advantages over production methods which utilized nontransgenic plants in which RGluc occurs naturally. Few RGluc-producing nontransgenic plants have been identified, and of these, many are not appropriate for general human or animal consumption. The present invention expands RGluc production to a wide variety of edible plants, resulting in (1) 20 increased sources for the procurement of RGluc producing plants; (2) a larger growing region for RGluc-producing plants which potentially leads to increased production; (3) reduction in seasonal and/or climate limitations associated with nontransgenic RGluc-producers; and (4) increased consumer satisfaction.

In the present invention, a plant which does not normally accumulate 25 stilbenes is transformed with a portion of a resveratrol synthase gene, leading to the uptake of a DNA fragment comprising the resveratrol synthase coding region from either a resveratrol synthase (RS) cDNA or genomic clone, and subsequent generation of RGluc in the plant. In the present invention, whole plants or tissue explants can be transformed. Transformed cells can be fully regenerated into

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intact plants or cultured as plant cell callus cultures, or plant cell suspension cultures.

In general, an RS coding region is selected. Resveratrol synthase cDNAs useful in the present invention can be obtained from mRNA isolated from any

5   natural source. For example, a cDNA from peanut can preferably be used to generate RGluc. Any plant known to produce resveratrol, RGluc, or other resveratrol derivatives may also serve as sources of suitable cDNAs, or coding sequences may be synthesized in vitro based on available sequences for resveratrol synthases. Coding regions may also be obtained from genomic DNA,

10   including or omitting intron sequences. Accumulation of RGluc is favored in plant tissues which contain the necessary biosynthetic precursors and low levels of β-glucosidases. Such conditions may be found in any plant cell, but are more likely to be present in non-stressed tissues, such as leaves constantly expressing RS driven by a constitutive promoter, rather than wounded or infected tissues

15   which contain, release, or induce β-glucosidases.

Preferably, a resveratrol construct is made by subcloning an RS cDNA into a promoter cassette. Any promoter compatible with the selected RS cDNA can be used in the vector constructs of the present invention. It is also believed that a promoter within the plant cell which is capable of initiating transcription of the RS

20   cDNA can be utilized, if the RS gene is integrated into the plant genome downstream of a transcription start site. Promoters which drive high levels of transcription in biosynthetically active plant cells during all stages of development are most likely to produce maximum levels of RGluc. For production of RGluc in plant tissues for direct human consumption, promoters must be selected to drive

25   expression in edible portions of the plants. Upstream (5') and downstream (3') untranslated sequences must also be included to ensure sufficient translation initiation and transcript processing (polyadenylation); these sequences can be derived from RS cDNAs or genomic clones, or from commonly available plant genes and transformation vectors.

The resulting transgene cassette is subsequently introduced into the plant cell where it is integrated into the plant cell genome. For *Agrobacterium*-mediated transformation, the resveratrol construct is subcloned into a binary vector. The resveratrol coding region and other elements of the construct will be

5   inserted between the right and left borders of the T-DNA region of the binary vector, i.e., the borders which define the DNA sequences that will be transferred from the *Agrobacterium* to the plant cells. For *Agrobacterium tumefaciens*-mediated transformation, leaf disc transformation and regeneration methods are preferred, but other methods such as vacuum infiltration of plants are also

10   possible. The same binary vectors can be used for *Agrobacterium rhizogenes*-mediated transformation, resulting in transformed cells, hairy roots, or regenerated plants. Other methods of introducing the transgene cassette into the living plant cell can be used, including but not limited to; biolistic particle bombardment, pollen transformation; protoplast electroporation; or permeabilization.

15   Several methods known in the art can be used to distinguish the progeny exhibiting stable inheritance of the transgene. For transgenic plants wherein the transgenic cassette contains both the RS cDNA and a gene coding for a visible phenotypic change, the selection can be based upon visual examination of the progeny. For plant transformations involving a selectable marker gene, the

20   appropriate selectable agent can be applied to the plants to select the transformants. Optionally, Southern blot analysis or PCR analysis can be used to verify the presence of the transferred gene in the genome of the transformed plants. RNA gel blot analysis, RT-PCR, or similar techniques can be used to verify the transcription of the RS gene in transformed tissues. Progeny which are

25   stably transformed with the RS construct and successfully accumulating RGluc can be identified by chemically analyzing the plant tissues for the presence of RGluc, using chemical methods including but not limited to organic extraction followed by high pressure liquid chromatography (HPLC), gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), or capillary

30   electrophoresis (CE).

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RGluc can be extracted from the plant tissue with organic solvents such as acetone or methanol or expressed from the tissues as a crude sap exudate. RGluc-containing plants, plant parts or cells can also be used in their fresh, frozen or dried form.

EXAMPLE 1: Transformation of Alfalfa with Peanut RS cDNA

Alfalfa was transformed using a peanut RS cDNA, and the resulting transgenic plants were investigated.

Plant material and greenhouse conditions

Alfalfa (*Medicago sativa* L.) c.v. Regen SY (Bingham, et al. 1991 "Regeneration of alfalfa hybrid Regen-Sy germplasm from tissue culture and transformation research," *Crop Sci* 31:1098) stocks and transgenic lines were maintained in greenhouse conditions under a 16 hour photoperiod in MetroMix 350 (The Scotts Co., Marysville, OH). Independent transgenic lines were vegetatively propagated by cuttings, planted in MetroMix 350 in growth chambers (Conviron, Winnipeg, Manitoba, Canada) under a 16 hour photoperiod with 100% relative humidity for 3 weeks.

Vector constructs and plant transformation

The peanut (*Arachis hypogaea*) resveratrol synthase (RS) cDNA (nucleotides 902 to 2854 of SEQ ID NO:1 and FIG. 1A and 1B) (Tropf, et al. 1994. "Evidence that stilbene synthases have developed from chalcone synthases several times in the course of evolution," *J Mol Evol* 38:610-618) was subcloned into the *Nco*I and *Bam*HI sites of a CaMV-35S dual enhancer promoter cassette pRTL2 (Restrepo, et al. 1990. "Nuclear transport of plant polyviral proteins," *Plant Cell* 2:987-998). The CaMV-35S:RS cassette was partially digested with *Hind*III (SEQ ID NO:1 and FIG. 1A and 1B) and subcloned into the *Hind*III site of the binary vector pGA482 (FIG. 2). Restriction enzyme digest and DNA sequencing confirmed the integrity of the construct. The binary vector and vector

constructs were maintained in *E. coli* DH5I_(Clontech, Palo Alto, CA) and transferred by electroporation to *Agrobacterium tumefaciens* LBA4404.

Transgenic alfalfa plants were generated from the transformation and regeneration-competent alfalfa (c.v. Regen SY) (Bingham, et al. 1991. *Crop Sci* 31:1098) following a modified version of published procedures (Bingham, et al. 1975. "Breeding alfalfa which regenerates from callus tissue in culture," *Crop Sci* 15:719-721). In the modified procedure, alfalfa plants were transformed with *Agrobacterium* strain LBA4404 harboring either the pGA482 (control) or the CaMV-35S:RS construct by leaf disc method with regeneration under kanamycin selection (Oommen, et al. 1994. "The elicitor-inducible alfalfa isoflavone reductase promoter confers different patterns of developmental expression in homologous and heterologous transgenic plants," *Plant Cell* 6:1789-1803). Surviving plantlets were placed on MS media without selection (Murashige, T. and Skoog, F. 1962. "A revised media for rapid growth and bioassay with tobacco tissue culture," *Physiol Plant* 15:473-497) for an additional month before transfer to Magenta boxes (Magenta Corp. Chicago, IL) and subsequent propagation by vegetative cuttings. Rooted cuttings were transferred to MetroMix 350, placed on a misting bench for one week, and finally placed under greenhouse conditions.

Southern blot and PCR analyses of transgenic lines

Genomic DNA was extracted from 1 gram of alfalfa green leaf tissue as described (Dellaporta, et al. 1983. "A plant DNA minipreparation: version II," *Plant Mol Biol Rep* 1:19-21). Twenty micrograms of DNA were digested with *Hind*III and size fractionated by 1% agarose gel electrophoresis (Sambrook, et al. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press). The gel was treated in 0.25 M HCl for ten minutes, denatured with 0.5 M NaOH/1.0 M NaCl for twenty minutes and neutralized with 0.5 M Tris-HCl/1.5 M NaCl for twenty minutes with gentle shaking and then transferred overnight by capillary action to a nylon membrane (GeneScreen Plus, DuPont, Boston, MA) in 5 x SSPE (1 x SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4). The DNA was covalently crosslinked to the membrane by

ultraviolet light (Stratalinker, Stratagene, La Jolla, CA). The hybridization probe
was prepared by digesting the pGA482 binary vector with *Bam*HI and *Hind*III to
release a fragment of *NPT*II gene. The gel-purified fragment was labeled with
^{32}P-dCTP via the random primer method (Prime a Gene, Promega, Madison, WI).

5   Southern hybridizations were carried out by the method of Church and Gilbert
(Church, G.M. and Gilbert, W. 1984. "Genomic sequencing," *Proc Natl Acad Sci
USA* 81:1991-1994) at 65°C. Independent transformants were subsequently
identified by the hybridization pattern. All independent transgenic plants
examined contained only one copy of the transgene cassette.

10   To identify those independent transgenic lines that successfully integrated
the full length coding region, RS cDNA specific primers 5'
CCATGGAAGGGGGAATTCGC 3' (SEQ ID NO:3) and 5'
GAGCCATTCAGCACCTTAGC 3' (SEQ ID NO:4) were used in PCR reactions.
One to two micrograms of genomic DNA were combined with 0.4µM (final

15   concentration) of primers, 10mM dNTPs, 1 unit of Taq-DNA polymerase
(Promega, Madison, WI), in 1X PCR reaction buffer adjusted to a final volume of
50 microliters. The binary vector construct used in the transformation protocol
served as a positive control reaction. Thermocycler (Singleblock System,
Ericomp, Inc., San Diego, CA) conditions were as follows: denature at 95°C for 2

20   minutes, anneal at 55°C for 1.3 minutes, elongate at 72°C for 2 minutes for a total
of 30 cycles, followed by a final elongation of 15 minutes at 72°C. Twenty five
microliters of PCR reaction products were separated by agarose gel
electrophoresis and visualized by ethidium bromide staining under UV light.

Northern blot analyses of RS and CHS transcript levels

25   Total RNA was extracted from the leaves, internodes or roots by the
method of Logemann, et al. (Logemann, et al. 1987. "Improved method for
isolation of RNA from plant tissues," *Anal Biochem* 163:16-20). After overnight
precipitation, the protocol was modified such that the RNA was purified from the
aqueous phase by the method of Zhou and Goldsbrough (Zhou, J. and

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Goldsbrough, P.B. 1993. "An Arabidopsis gene with homology to glutathione S-transferase is regulated by ethylene," *Plant Mol Biol* 22:517-523). The RNA (10 µg/lane) was size fractionated by 1% agarose-formaldehyde gel electrophoresis by the method of Sambrook, et al. (Sambrook, et al. 1989. *Molecular Cloning: A*

5 *Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press). The RNA was transferred overnight by capillary action to a nylon membrane (GeneScreen Plus, DuPont, Boston, MA) in 5X SSPE. The hybridization conditions and radio-labeling of probes were prepared as described in the previous section. For individual experiments, probes included a resveratrol synthase cDNA from peanut

10 (Tropf, et al. 1994. *J Mol Evol* 38:610-618), a chalcone synthase cDNA (Junghans, et al. 1993. "Stress responses in alfalfa (*Medicago sativa* L.) 15. Characterization and expression patterns of members of a subset of the chalcone synthase multigene family," *Plant Mol Biol* 22:239-253) and hybridized with the plant 18S ribosomal RNA probe (Eckenrode, et al. 1985. "Comparison of the

15 nucleotide sequence of soybean 18S rRNA with the sequences of other small-subunit rRNAs," *J Mol Evol* 21: 259-269) as a loading control.

High levels of RGluc accumulate in the young leaves (approximately 150 µg/g fresh weight) and in the old internodes (approximately 130 µg/g fresh weight) of the highest accumulating alfalfa lines expressing RS. However, HPLC

20 analyses of root extracts detected only marginal levels of RGluc (<2.0 µg/gram fresh weight) in these same independent lines (data not shown). Alfalfa roots are known to accumulate high constitutive levels of flavonoids and isoflavonoids (Oommen, et al. 1994. *Plant Cell* 6:1789-1803), and CHS would be a direct competitor of metabolic intermediates p-coumaroyl CoA and malonyl CoA.

25 Therefore, northern blot experiments were performed to compare the relative transcript levels of RS and CHS in the leaves, internodes and roots of three independent CaMV35S:RS lines and a vector control line. Results in FIG. 3A and FIG. 3B show that the highest levels of RS transcripts are found in the leaves and internodes while comparatively low levels were detected in the roots (lanes 1-3).

30 As expected, no RS transcripts were detected in the vector control (lane C)(FIG. 3A). In contrast, the highest CHS message levels were detected in the roots

compared to the leaves and internodes (FIG. 3B). Therefore, the nearly undetectable levels of RGluc in the roots are due to a combination of relatively low RS transcript levels and strong competition for the available substrate pool of high levels of CHS.

5   **Characterization and RGluc identification by HPLC analysis**

Independent transgenic alfalfa lines harboring the CaMV35S:RS construct were analyzed for the presence of resveratrol by reverse phase C18 HPLC analyses (4.6 X 250mm column, Baker, Phillipsburg, NJ). Metabolites were extracted from 0.5–1.0 grams of fresh leaves, internodes or roots in 100% acetone

10   for up to two days. The supernatant was dried completely under nitrogen and dissolved in 0.7 ml of methanol followed by 0.3 ml water. The sample was vortexed vigorously, sonicated for 5 minutes and then centrifuged to remove the insoluble debris. The final supernatant was concentrated to dryness then dissolved in 0.1 ml of methanol. For analysis, 20 μl of sample was injected and metabolites

15   separated by a 45 minute linear gradient from 20–60% solvent B (solvent A = 1.0% H_3PO_4, solvent B = 100% acetonitrile) with a flow rate of 0.8 ml/min monitored at λ=320nm. UV spectra of peaks of interest were recorded with a UV diode array detector (Beckman Instruments, Fullerton, CA). Characterization of the putative resveratrol-conjugate was performed by β-glucosidase digestion of a

20   crude leaf extract. The methanol was removed under nitrogen and the residue dissolved in 200μl of 25mM citric acid/phosphate buffer (pH=5.2) containing 0.5 mg/ml β-glucosidase from almonds (Sigma Chemical Co., St. Louis, MO). After incubation at 37°C for 1 hour, the free resveratrol was extracted from the aqueous phase 3 times with an equal volume of ethyl acetate. The ethyl acetate was pooled

25   and concentrated to dryness under nitrogen, dissolved in methanol, and then analyzed by HPLC. The identity of resveratrol was confirmed by co-chromatography with a resveratrol standard (Sigma) and by comparison of UV absorbance spectra.

Independent transgenic alfalfa lines harboring the CaMV35S:RS construct
30   as evidenced by Southern blot and PCR analyses were analyzed by HPLC for the

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constitutive accumulation of resveratrol. Results shown in FIG. 4 reveal the
presence of an unknown peak (8.2 minutes) that appears only in the leaves and
internodes of lines transformed with the CaMV35S:RS binary vector
(chromatogram A) and not vector control plants (chromatogram B). The UV

5   spectra analysis of the 8.2 minute peak and a resveratrol standard are nearly
identical with absorbance maxima of 307 nm and 320 nm for both compounds
(FIG. 5). However, the 8.2 minute retention time is significantly less than the
retention time observed with a resveratrol standard (14.9 minutes). This result
shows that the resveratrol had been modified by the addition of hydrophilic

10   moiety, possibly a sugar. Hydrolysis with β-glucosidase of the unknown
compound results in the appearance of free resveratrol (FIG. 6). In addition,
removal of H_3PO_4 from the aqueous phase of HPLC solvent system did not alter
the retention time of the resveratrol conjugate (data not shown). This observation
shows that the sugar moiety was not substituted with a charged group such as

15   malonic acid.

^{1}H and ^{13}C NMR analyses of RGluc

The suspected resveratrol glucoside conjugate was purified from several
kilograms of transgenic alfalfa leaves and internode tissue. Large volumes of
crude acetone extracts were concentrated to near dryness under vacuum.

20   Insoluble materials were removed by centrifugation with the remaining
supernatant adjusted to 50% methanol. The extract was passed over C18 reverse
phase disposable cartridge (SepPak, Waters Corp, Milford, MA) followed by
concentration to near dryness under vacuum to remove the methanol. The
aqueous phase was extracted 3 times with an equal volume of ethyl acetate. The

25   ethyl acetate fractions were pooled and concentrated to dryness under vacuum.
The final residue was dissolved in methanol. The resveratrol-conjugate was then
purified by preparative C18 reverse phase HPLC (22.5 x 250mm, Econosil,
Deerfield, IL). For purification, 0.5 ml of extract was separated by a 45 minute
linear gradient from 10-25% solvent B (solvent A = water, solvent B =

30   acetonitrile) at a flow rate of 20 ml/minute monitored a λ = 320nm. Fractions

containing the resveratrol conjugate were pooled, concentrated to dryness under
nitrogen and purified a second time. The final fraction was concentrated to
dryness and dissolved in a small volume of methanol. The concentration was
estimated by comparison of the absorbance spectra to a known concentration of

5   the resveratrol standard. The sample to be analyzed was dried under nitrogen and
analyzed by ^{13}C NMR (150MHz, CD$_3$OD) and ^{1}H NMR (600 MHz, CD$_3$OD).
Identity was confirmed by comparison to previously published reports (Mattivi, et
al. 1995. *J Agric Food Chem* 43:1820-1823; Waffo-Teguo, et al. 1998. *J Nat Prod*
61:655-657).

10   Data obtained from ^{13}C-NMR and ^{1}H-NMR given in Tables I and II below
were highly consistent with previous reports which identified this compound as
trans-resveratrol-3-O-β-glucopyranoside, commonly known as piceid or
polydatin (Waterhouse, A.L. and Lamuela-Reventost, R.M. 1994. "The
occurrence of piceid, a stilbene glucoside in grape berries," *Phytochemistry*

15   37:571-573; Mattivi, et al. 1995. *J Agric Food Chem* 43:1820-1823; Waffo-
Teguo, et al. 1998. *J Nat Prod* 61:655-657). Table I contains ^{13}C NMR values for
the RGluc purified from transgenic alfalfa (in column RS-X) compared with
published values for resveratrol glucoside purified from two non-transgenic plants
(Waffo Teguo, et al. 1998. "Isolation, identification, and antioxidant activity of

20   three stilbene glucosides newly extracted from Vitis vinifera cell cultures," *J Nat
Prod* 61:655-657; and Mattivi, et al. 1995. "Isolation, characterization, and
evolution in red wine vinification of resveratrol monomers," *J Agri Food Chem*
43:1820-1823). Table II contains proton NMR values for the RGluc purified from
transgenic alfalfa (in column RS-X) compared with published values for

25   resveratrol glucoside purified from a non-transgenic plant (Waffo Teguo, et al.
1998. *J Nat Prod* 61:655-657).

In preliminary experiments, HPLC analyses of shoots collected from
independent transgenic alfalfa lines found that the concentrations of RGluc ranged

Table I: Comparison of ^{13}C-NMR Chemical Shift Values
(150 MHz, CD$_3$OD)for Isolated Resveratrol Conjugate
with Values from Two Published Studies

C[a]	δ (RGluc)	δ [b]	δ [c]
1	141.43	141.4	140.9
2	107.05	107.0	106.2
3	160.47	160.5	160.2
4	104.13	104.1	103.9
5	159.58	159.6	159.4
6	108.38	108.4	108.2
7	126.69	126.7	126.5
8	129.99	130.0	129.9
1'	130.34	130.3	130.0
2',6'	128.91	128.9	128.9
3',5'	116.50	116.5	116.4
4'	158.47	158.5	158.2
Glucose			
1"	102.43	102.4	102.1
2"	74.97	75.0	74.8
3"	78.07	78.1	78.1
4"	71.51	71.5	71.5
5"	78.25	78.3	77.8
6"	62.62	62.6	62.8

[a] Carbon assignments as reported by Mattivi et al.

[b] Values reported by Teguo et al. (1996).

[c] Values reported by Mattivi et al. (1995).

Table II: Comparison of ^{1}H-NMR chemical shift values
(600 MHz, CD_3OD) for the isolated resveratrol
conjugate with published values

Proton Assignment (Teguo)[a]	δ(RGluc)	δ[b]
H-2', H-6'	7.35 (2H, d)	7.35 (2H, d)
H-8	7.01 (1H, d)	7.00 (1H, d)
H-7	6.84 (1H, d)	6.84 (1H, d)
H-2	6.78 (1H, br s)	6.78 (1H, br s)
H-3', H-5'	6.76 (2H, d)	6.76 (2H, d)
H-6	6.61 (1H, br s)	6.61 (1H, br s)
H-4	6.45 (1H, br s)	6.44 (1H, br s)
Glc H-1"	4.89 (1H, d)	4.88 (1H, d)
Glc H-6a"	3.92 (1H, dd)	3.92 (1H, dd)
Glc H-6b"	3.71 (1H, dd)	3.70 (1H, dd)
Glc H-2", H-3", H-4", H-5"	3.48 - 3.38 (4H, m)	3.48 - 3.38 (4H, m)

[a] Proton assignments as reported by Teguo et al.(1996)

[b] Values reported by Teguo et al. (1996).

from 5.0 - 20.0 µg/gram fresh weight, measured as resveratrol equivalents. However, these data were significantly variable for replicate samples taken from the same independent lines. Therefore, an experiment was performed to test whether the concentration of the RGluc was developmental or tissue dependent. Independent alfalfa lines that showed consistently high levels of RGluc were grown under greenhouse conditions. The leaves and internodes from several shoots were harvested, pooled and extracted for HPLC analysis according to their relative age and position on individual alfalfa shoots For example, L-1 was considered the youngest leaf (FIG. 7). Results show that the concentration of RGluc in the leaves declines significantly from the youngest to the oldest leaves on a µg/gram fresh weight basis (FIG. 8). In contrast, the concentrations increase significantly from the youngest internodes to the oldest. Similar results were

obtained in two additional RGluc-accumulating lines (data not shown). Northern blot analyses taken from the same independent line indicated that the relative level of RS transcripts were not significantly affected by the age or tissue type (FIG. 9). Taken together, these data demonstrate there is no correlation between RS

5   transcript levels and extractable levels of RGluc in these tissues. The concentration of RGluc in these tissues is dependent upon the relative availability of metabolic intermediates, p-coumaroyl CoA and malonyl CoA.

Agar-plate bioassay and plant-pathogen interactions

The alfalfa fungal pathogen *Phoma medicaginis* Malbr. & Roum. var.

10   *medicaginis* (Leath, K.T.,"Spring black stem and leaf spot." In *Compendium of Alfalfa Diseases*, D.L. Stuteville & DC Erwin, 2nd ed., American Phytopathological Society Press, 1990, p.16-17) maintained on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI) for one month in a dark growth chamber at 22°C. Agar-plate bioassays were performed by dissolving resveratrol

15   or RGluc purified from transgenic alfalfa lines in 50 µl of ethanol. The concentration of RGluc (measured in resveratrol equivalents) was estimated by HPLC analysis and comparison to a known concentration of a resveratrol standard. The ethanol solution was added to molten PDA such that the final molar concentrations of resveratrol or RGluc were 0.18 mM (50µg/ml resveratrol or 85

20   µg/ml for RGluc). For this experiment, control plates were made whereby an equal volume of ethanol was added to PDA without resveratrol. A small agar plug of *P. medicaginis* was placed in a petri dish containing the resveratrol-PDA mixture, sealed with parafilm and placed in a dark grown chamber at 22°C. The extent of hyphal growth was measured after one week.

25   To test for resistance in the transgenic lines, the first and second trifoliate leaves of greenhouse grown transgenic alfalfa lines were wound inoculated with a 27.5 gauge syringe needle dipped in a *P. medicaginis* spore suspension. The number of colony forming units per ml (CFU/ml) was estimated by plating a serial dilution of the spore suspension on PDA. After inoculation, the stem was cut at

30   the fourth internode and placed in a magenta box, with the cut end imbedded in

0.8% water agar (Phytagar, Difco, Detroit, MI). The magenta box was sealed with parafilm to maintain 100% humidity and placed in a growth room (16 hour photoperiod, 22°C) where disease symptoms were allowed to develop for 8 to 10 days. The extent of necrosis around the wound sites was measured with a digital video imaging system (Ultra-Lum, Inc., Paramount, CA) and analysis software (Scion Image for Windows, Frederick, MD).

To determine the extent of hyphal growth and the formation of reproductive structures, excised leaves were place in 100% humidity for up to three days followed by staining with a trypan blue/ethanol solution (Keogh, et al. 1980. "Comparison of histological and physiological responses to *Phykospora pachyrhizi* in resistant and susceptible soybeans," *Trans Br Mycol Soc* 74:329-333). Briefly, the leaves were completely covered with the trypan blue staining solution (10 ml lactic acid; 10 ml Tris-equilibrated phenol, pH 7.4; 10 ml glycerol; 10 ml water; 10 mg trypan blue; Sigma) followed by an equal volume of 100% ethanol. The samples were boiled for five minutes, and then allowed to cool at room temperature for up to 24 hours. For destaining, the staining solution was discarded, 0.8 g/ml of chloral hydrate was added followed by boiling for three minutes. The chloral hydrate was discarded, and the leaves rinsed well with water and stored in 50% glycerol solution. Fungal hyphae and pycnidia were examined with a dissecting microscope (Model SMZ-10, Nikon, Japan) and photographed with Ektachrome 160T color slide film (Eastman Kodak, Rochester, NY) with a Nikon FX-35WA 35mm camera.

Prior to the transformation experiments, agar-plate bioassays were used to test a resveratrol standard for anti-fungal activity against several alfalfa pathogens. Results showed that an isolate of *Phoma medicaginis* was the most sensitive (data not shown). RGluc was purified by preparative HPLC from transgenic lines and tested in agar plate bioassays in order to assess its effectiveness against this same isolate. Results showed that hyphal growth was significantly reduced (>50%) in the presence of RGluc (85 µg/ml) or resveratrol (50 µg/ml). Three replicates of this experiment were performed. Importantly, the concentration of RGluc

determined by HPLC analysis of the transgenic alfalfa lines is significantly higher to that used in these bioassays. Therefore, a sufficient concentration of RGluc should be available to inhibit pathogen ingress and reduce disease symptoms.

5   Two independent alfalfa lines that consistently accumulate the highest levels of RGluc and two independent pGA482 vector control lines were tested for resistance to *P. medicaginis*. First (L1) and second trifoliate (L2) leaves (FIG. 7) were wound-inoculated with small gauge needles that were dipped in spore suspensions varying from 10^4 to 10^7 colony forming units (CFU/ml). After ten days, the brown necrotic zone surrounding the wound site was measured. Results

10   in FIG. 10 and FIG. 11 show that over the range of CFU's tested, the area (mm^2) of tissue necrosis is reduced on average by as much as 50% as compared to the vector control plants. In addition, the observed area of chlorosis surrounding the necrotic zone also appeared to be greatly reduced. (FIG. 12A and FIG. 12B).

Further evidence of increased resistance to *P. medicaginis* was found after

15   the detached leaves that appeared to be the most resistant (10^4 CFU/ml; FIG. 10 and FIG. 11) were allowed to incubate at 100% relative humidity. After three days, the leaves were stained with trypan blue in order to visualize the extent of hyphal ingress. Results in FIG. 12C show extensive hyphal growth in the vector control line and the formation of reproductive structures (pycnidia). In contrast,

20   only a small area of fungal hyphae (blue staining) outside of the necrotic zone could be observed in inoculated leaves from a CaMV35S:RS transgenic line (FIG. 12D). Subsequently, the extent of hyphal ingress outside of the necrotic zone surrounding the wound site and the number of pycnidia were measured. Results in FIG. 13 show the extent of hyphal ingress and number of pycnidia formed

25   beyond the necrotic zone is much greater in the vector control lines than in the CaMV35S:RS lines. In only seven out of fifty-seven lesions examined from the CaMV35S:RS lines could pycnidia be identified, compared to forty-seven out of fifty lesions from the vector controls. Together these experiments demonstrate that the accumulation of RGluc is sufficient to reduce the extent of leaf necrosis,

prevent chlorosis caused by hyphal ingress and reduce the number of reproductive structures.

EXAMPLE 2: *Agrobacterium*-mediated Transformation of Soybean

In soybean, the CaMV-35S:RS binary construct or the pGA482 binary

5   vector control plasmid was introduced into *Agrobacterium rhizogenes* Strain K599 (Savka, et al. 1990. *Phytopathology* 80:503-508) using a standard freeze-thaw technique. *A. rhizogenes* colonies containing the binary vector were selected on YMB agar plates with kanamycin (25 mg/l) and tetracycline (12 mg/l). The composition of the YMB agar plates per liter was 0.45 grams anhydrous dibasic

10   potassium phosphate, 0.2 grams magnesium sulfate ($7H_2O$), 0.1g sodium chloride, 10 g mannitol, and 0.4g yeast extract. Components were dissolved in distilled water, the pH adjusted to 6.5, 15 g of bacteriological grade agar (Bactoagar, Gibco, Rockville, MD) was added, and the mixture was sterilized by autoclaving for thirty minutes at 121°C. After cooling to approximately 50°C, the appropriate

15   antibiotics were added and poured into sterile plastic petri plates. Selected colonies were re-streaked onto fresh plates and used for inoculum.

Soybean seeds (*Glycine max* cv. Harosoy 63) were surface sterilized with 70% ethanol for ten minutes and 20% bleach at a final concentration of approximately 1% sodium hypochlorites for twenty minutes, and then rinsed three

20   times with sterile distilled water. Surface sterilized seeds were then placed on sugar-water-agar (SWA) plates for germination at 23-25°C with 16 hour light/8 hour dark, with cool-white fluorescent bulbs providing illumination. SWA contains 0.8% Gibco tissue culture grade Phytagar, 0.5% sucrose in distilled water which was autoclaved for 30 minutes, cooled to 50°C, and then poured into 150

25   mm by 25 mm transparent plastic Petri dishes or pre-sterilized Magenta boxes.).

The cotyledons of nine to eleven-day-old soybean seedlings were wounded using a sharp scalpel dipped into the *Agrobacterium rhizogenes* mass growing on the surface of the YMB plates described above. The seedlings were transferred to transparent plastic Magenta boxes (Magenta Corp, USA, Chicago, IL) containing

SWA medium, with roots embedded in the medium to hold the seedlings upright. The first morphogenic change, detected at 5-10 days after inoculation, was callus formation at the inoculation sites. Within two weeks, the first roots were visible; they grew directly out of the callus. The number of roots produced varied from

5   four to nine per inoculated cotyledon after 3 weeks. Plants wounded with a sterile scalpel (uninoculated control) did not form roots at the wound site.

Approximately 50 to 60% of the emerging hairy roots were "co-transformed" (contain both the Ri DNA from the genome of the *A. rhizogenes* and the T-DNA region of the introduced binary vector). Individual hairy roots were

10   transferred to Schenk & Hildebranbt (SH) agar medium supplemented with phytohormones (Oommen et al. 1994. *Plant Cell* 6:1789-1803) and carbenicillin (500 mg/ml) to initiate callus from the roots and to eliminate any *A. rhizogenes*. The hairy roots and newly initiated calli were transferred to fresh medium at weekly intervals.

15   Four to five weeks after initiation, calli were aseptically sampled (100mg) and analyzed for the presence of the NPT II gene using an NPT II ELISA kit (5Prime3Prime Inc., Boulder, CO). Calli induced by *A. rhizogenes* with no binary vector were negative for NPT II ELISA. Calli positive by this ELISA assay contained at least part of the T-DNA region from the binary vector. After

20   further increases in the calli mass, these positive calli (and representative non-transformed calli) were then assayed for the presence of RGluc and the presence of the CaMV-35S:RS construct, using the techniques similar to those used for the transgenic alfalfa. From each pGA482 vector control and CaMV-35S:RS transgenic callus line, approximately 25 grams of soybean callus cells were

25   aseptically removed from the culture dish, and submerged in 150 ml acetone to extract the phenolic compounds. After two days shaking, the mixture was centrifuged, the acetone extract taken to dryness on a rotary evaporator unit, and the residue dissolved in methanol. After centrifugation, this extract was analyzed by HPLC, as described for the alfalfa extracts.

Stilbenes have not been reported to accumulate in soybean plants, seeds, cell cultures or food products. No RGluc was observed in the control (nontransformed soybean callus extracts). A new peak was present in the HPLC chromatograms for CaMV-3S:RS transformed calli. This new compound was

5   identified as RGluc, based on the observation that its properties were identical to the RGluc from alfalfa (identical HPLC retention time, lack of change in retention time which mobile phase component is changed from 1% H_3PO_4 to pure H_2O, identical UV diode array scan, UV scan similar to free resveratrol, ability to be hydrolyzed to free resveratrol by beta-glucosidase, etc.)(FIG. 14). FIG. 14 shows

10   the HPLC chromatogram of a transgenic soy callus with a RGluc peak at 27 minutes. FIG. 15 shows the HPLC chromatogram of a non-transgenic soy callus line. To enhance the resolution of the RGluc from the endogenous soybean cell phenolics in crude extracts as in FIG. 14, the gradient was altered from that used for alfalfa plants extracts. The gradient for crude soy extracts was 90%

15   water/10% CH_3CN initially with a linear change to 25% CH_3CN in 45 minutes. FIG. 16 is the UV diode array scan of the soybean callus-generated RGluc peak, which matches closely the alfalfa-generated peak (FIG. 5). FIG. 17 shows the RGluc partially purified from soy callus before treatment with 1 mg beta-glucosidase, and FIG. 18 shows the RGluc partially purified from soy callus after

20   treatment with 1 mg beta-glucosidase (from almonds; Sigma, St. Louis) for 45 minutes in pH5.5 citrate/phosphate buffer. Samples in FIG. 17 and FIG. 18 were generated using the same extraction, purification, and HPLC analytical methods as were used for the transgenic RGluc alfalfa studies. RGluc was eluted at approximately 8 minutes and the resveratrol was eluted at approximately 15

25   minutes.

These transgenic plant cell callus cultures, or those of a similarly transformed species, can be grown axenically for years as callus or as cell suspension cultures to produce large quantities of RGluc-containing cells for direct use, or for the production of extracts containing RGluc, or for the

30   production of purified RGluc.

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EXAMPLE 3: RGluc-producing Transgenic Alfalfa Diet for Mouse

Because alfalfa is a highly nutritious animal feed, a study was carried out to compare the chemopreventative activity of a basal diet and a diet supplement with dried transgenic RGluc accumulating alfalfa.

5   The transgenic alfalfa was dried for 24-48 hours in a convection oven, powdered in a cyclone grinding mill to a particle size of less than one millimeter, and then blended at a rate of 20% weight/weight into the basic diet, which was then formed into food pellets. Four replicate samples of the dried powdered alfalfa was extracted with 90% acetone/10% water for a 24 hour period. The

10   extract was taken to dryness and resuspended in methanol and subjected to HPLC analysis for RGluc. The powdered alfalfa contained 162 micrograms RGluc (equivalent on a molar basis to approximately 95 micrograms resveratrol).

The assay used was the mouse aberrant crypt foci (ACF) system (Olivo, S. and Wargovich, M.J. 1998. "Inhibition of aberrant crypt foci by chemopreventive

15   agents," *Vivo-Attiki* 12:159-166.). Mice were injected with varying levels of tumor-inducing compound azoxymethane (AOM; 10 mg/kg body weight weekly) for five weeks while being fed basal diet, the mice were then fed test diets for five weeks, and the number of putative precancerous lesions in the colon (ACF) in the colon were subsequently recorded. Mice in the group receiving the RGluc-alfalfa

20   supplemented diet consumed approximately $4.8 \pm .2$ grams per day, which indicates an average dose of 155 micrograms of RGluc per day (or 91 micrograms resveratrol per day), which translates to approximately 4.7 mg RGluc per kg body weight (or 2.8 mg resveratrol per kg body weight). Preliminary experiments indicate that supplementation of the basal diet with 10 and 20% (weight/weight)

25   powdered alfalfa did not significantly affect the body weight or food consumption, indicating that the diets were well matched in nutritional qualities and caloric content.

Mice receiving the control diet achieved an average body weight of 31.3 g $\pm$ 1.3 g and were found to have an average of 30.8 ± 10.2 foci/colon. Mice

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receiving the RGluc-alfalfa supplemented diet achieved an average body weight of 32.8 g $\pm$ 2.2 g and were found to have an average of 21.6 $\pm$ 5.5 foci/colon. Compared to ACF numbers in mice fed a basal (control) diet, a significant reduction in ACF number was noted when mice were fed a diet supplement with

5   dried transgenic RGluc accumulating alfalfa. These results indicate that the a diet supplemented with dried transgenic RGluc accumulating alfalfa inhibited colon carcinogenesis in mammals.

It is to be understood that the above description is of preferred exemplary embodiments of the invention and is intended to be illustrative of the invention,

10   but is not to be construed to limit the scope of the invention in any way. Modifications may be made in the structural features of the invention without departing from the scope of the invention. It will be readily apparent to those skilled in the art that alternative materials may also be utilized without departing from the scope of the invention.